

# Effect of raloxifene on alveolar bone resorption after mucoperiosteal flap surgery in mice

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## ABSTRACT

**Background:** Mucoperiosteal flap surgeries (MFS) are carried out to provide access to the alveolar bone and root surfaces in several clinical situations. Nevertheless, they lead to a variable degree of alveolar bone resorption. Raloxifene is an agonist in bone, and acts inhibiting bone loss.

**Objective:** To evaluate the effect of raloxifene in preventing alveolar bone resorption after MFS using an experimental model of mouse mandibles.

**Methods:** MFS was performed on the buccal aspect of the left side of the mandible (BL) in 20 male CF1 *Musdomesticus* mice divided into two groups with the same number of animals: the experimental group was treated once daily with raloxifene injections (3 mg/kg), and the placebo group was treated with daily injections of the vehicle. The buccal aspects of right hemimandibles were used as controls (BR). Mandibles were removed, defleshed and stained with toluidine blue in a stereomicroscope. Digital images were obtained and the alveolar bone loss was measured (mm<sup>2</sup>) using an image analysis software.

**Results:** The BL area exhibited significantly more bone loss (Student *t* test;  $p < 0.01$ ) when compared to the BR area, in both groups. No statistically significant difference was observed between the experimental and the placebo groups.

**Conclusion:** In this study, raloxifene did not inhibit alveolar bone resorption following MFS in male mice.

**Keywords:** Raloxifene Hydrochloride; Alveolar Bone Loss; Surgery, Oral.

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# **Efeito do raloxifeno sobre reabsorção óssea alveolar após cirurgia de retalho mucoperiosteal em camundongos**

## **RESUMO**

**Introdução:** Cirurgias de retalho mucoperiosteal (CRM) são realizadas para permitir acesso ao osso alveolar e à superfície radicular em várias situações clínicas. No entanto, elas levam a um grau variável de reabsorção óssea alveolar. O raloxifeno tem ação agonista em tecido ósseo e atua inibindo perda óssea.

**Objetivo:** Avaliar o efeito do raloxifeno na prevenção de reabsorção óssea alveolar após CRM usando o modelo experimental de mandíbulas de camundongos.

**Métodos:** Foram realizadas CRMs na face vestibular, lado esquerdo, das mandíbulas (VE) de 20 camundongos CF1 *Musdomesticus* machos, divididos em dois grupos com mesmo número de animais: o grupo experimental foi tratado uma vez ao dia com injeções de raloxifeno (3 mg/kg), e o grupo placebo foi tratado uma vez ao dia com injeções do veículo. A face vestibular do lado direito da hemimandíbula foi usada como controle (BD). As mandíbulas foram removidas, dissecadas e coradas com azul de toluidina sob um estereomicroscópio. Imagens digitais foram obtidas e a perda óssea alveolar foi medida (mm<sup>2</sup>) usando um software de análise de imagens.

**Resultados:** A área VE exibiu perda óssea significativamente maior (teste *t* de Student; *p* < 0,01) quando comparada com a área BD, em ambos os grupos. Não foi observada diferença estatisticamente significativa entre os grupos experimental e placebo.

**Conclusão:** Neste estudo, o raloxifeno não inibiu a reabsorção óssea alveolar após CRM em camundongos machos.

**Palavras-chave:** Cloridrato de Raloxifeno; Perda do Osso Alveolar; Cirurgia, Oral.

## **INTRODUCTION**

Bone is a dynamic tissue with high remodeling capacity in response to the body's metabolic requirements. Under normal circumstances, bone volume and mass are held constant by bone resorption and apposition processes (1). Nevertheless, some factors, including surgical bone exposure, may lead to some imbalance in these mechanisms.

In dentistry, surgical access to the alveolar bone is routinely conducted as part of different treatment approaches, including restoration, periodontal treatment, rehabilitation, endodontic treatment, and in the surgical removal of teeth and/or pathological processes. During the dissection process, the periosteum is detached from the alveolar bone, leading to a resorptive phase due to the stimulation offered by osteoclastic activity and loss of bone crest (2-4). In rodent mandibles, resorption is also observed after surgery (4,5).

Alveolar bone volume reduction may interfere with the success of oral rehabilitation treatments. Therefore, loss of this bone tissue should be prevented (6). The use of systemic or local drugs in the prevention of bone resorption has been studied using animal models (7-16).

Raloxifene, a selective estrogen receptor modulator, is an agonist to the human skeleton. The compound has been shown to act as an antiresorption drug by reducing biochemical markers of bone remodeling and preserving bone mineral density in the lumbar spine and femur neck, with significant reduction in the occurrence of new fractures. In addition, raloxifene is an antagonist to endometrial and mammary tissues (17,18). The preventive effect of raloxifene on the expression of selective bone turnover markers has also been observed during the alveolar healing process in female rats submitted to ovariectomy surgery (15,19,20).

This study aims to evaluate the effect of raloxifene in preventing alveolar bone resorption after mucoperiosteal flap surgery (MFS) using an experimental model of mouse mandibles (5).

## **MATERIALS AND METHODS**

This study was approved by the Research Ethics Committee of Universidade Luterana do Brasil (ULBRA).

A total of 20 3-month-old male CF1 mice (*Musdomesticus*) weighing 30 g on average were used in this study. Animals were obtained from the colonies maintained by state-owned research institution Fundação Estadual de Produção e Pesquisa em Saúde. Mice were randomly divided into two experimental groups, consisting of 10 mice each, all undergoing MFS. The first group (experimental group, EG) was treated with raloxifene injections; the second group (placebo group, PG) was given injections of the vehicle used in raloxifene dilutions.

Animals were maintained in individual sterilized plastic cages (Beira Mar, São Paulo, SP, Brazil) with an iron cover and solid bottom during the experiment. Sterilized bedding (Vet-Sul, Porto Alegre, RS, Brazil) was provided. Mice consumed standard food (Nutrival, CR-1, Nutrival Nutrients, Curitiba, PR, Brazil) and distilled water *ad libitum*. Standard conditions of light (12-hour light/12-hour dark cycle) and temperature ( $\square 20^{\circ}\text{C}$ ) were kept during the experiment. The experiment was conducted under proper ventilation and all animals were monitored daily.

### **Experimental procedure**

Initially, mice of both groups were weighed and anesthetized using intramuscular ketamine 100 g/L (Dopalen, AgribRANDS do Brazil, Paulina, Brazil) + 2% aqueous solution of 2-(2,6-xilidine)-5,6-dihydro-4-H-1,3-thiazine hydrochloride (Rompun, Bayer S.A., São Paulo, Brazil) at a 1:1 ratio in a dosage of 1.0 mL/kg. The mucosa was separated from the underlying bone after an incision at the marginal gingiva with a small elevator and then immediately readapted without any suture (4,5,7,10-13). The mucoperiosteal flap was performed on the buccal aspect of the left side of the lower left molars in a procedure that took approximately 40 seconds; the right side was used

as control (CG). Animals received only water during the initial 24 hours, to avoid displacement of the flap.

Mice in the EG were treated with subcutaneous injections of raloxifene (Raloxifenchloridrate, Eli Lilly, Brazil) (3mg/kg) dissolved in 100  $\mu$ L of olive oil, daily, throughout the experimental period. The PG received the vehicle (100  $\mu$ L olive oil/mice) (Figueira da Foz, Portugal), also daily (8,10). Animals were weighed daily before the injections to calculate the correct dose. Twenty-one days after the surgical procedure, animals were euthanized by means of cervical dislocation (4) under anesthesia.

Under a surgical stereomicroscope (M900, D F Vasconcelos, São Paulo, Brazil), mandibles were sectioned in the midline, defleshed, and all the organic material was removed with the help of sodium hypochlorite (Biodinâmica Química e Farmacêutica Ltda., Ibioporã, Brazil). Specimens were then stored in 10% buffered formalin for 12 hours (21-23). Each hemimandible was stained with 1% toluidine blue to disclose the root-exposed area. Limits of enamel, cementum and evident bone were observed in a stereomicroscope (Stemi SV6, Carl Zeiss, Inc., USA) under magnification (x3.2), using standardized position (24) and light. Images were digitally captured (Pixera Professional, Pixera, San Jose, USA).

### **Bone loss analysis**

Bone loss was measured by a single blind, calibrated examiner using image analysis software (Image Tool, UTHSCA, Texas, USA). Two independent measurements were conducted with an interval of 1 week between them, in order to allow reproducibility analysis. In the present study, intra-examiner and trans-experiment reproducibility values (95% confidence interval) were represented by the means of differences between the pairs of measurements of alveolar bone loss (ABL), and ranged between -0.03 mm<sup>2</sup> and 0.03 mm<sup>2</sup>.

In the buccal aspect, the area of the ABL was measured in the first molar, whereas in the lingual aspect, the area of ABL was measured in the first and second molars using the reference points proposed by Tatakis and Guglielmoni (23) and modified by Hilgert et al. (25). The ABL area in the lingual aspect was defined as follows: mesially, by the mesial edge (cemento-enamel junction [CEJ] to the alveolar bone) of the mesial root of the first molar; distally, by the distal edge (CEJ to the alveolar bone) of the distal root of the second molar; coronally, by three points on each of the two molar teeth (two points defined by the position of the CEJ on the mesial and distal aspects of the tooth and one defined by the most apical position of the CEJ on the tooth surface); apically, by the most apical position of the alveolar bone on the first and second root surfaces (Figure 1). Results are presented in square millimeters (mm<sup>2</sup>).

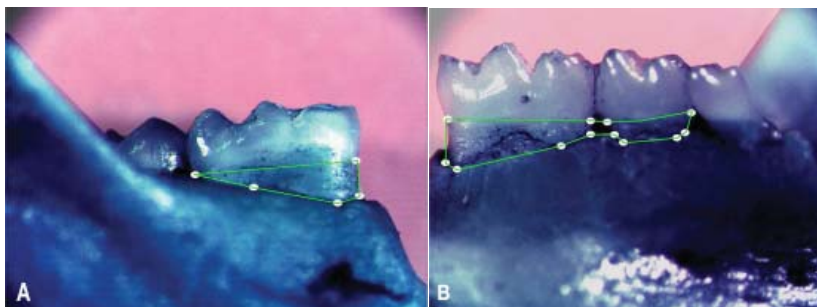


FIGURE 1 – Landmarks used to define the alveolar bone loss area. Connecting all points together represents the area of interest. A) Left side, buccal aspect; B) Left side, lingual aspect.

## Statistical analysis

The Student *t* test for independent samples was used to assess the presence of significant differences in respect to alveolar bone loss between mice treated with raloxifene and control mice. Furthermore, the Student *t* test was also used to check for intragroup differences (e.g., in BL and BR ABL areas of mice within each group). The value set to reject the null hypothesis was  $p \leq 0.05$ . All analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 12.0 for Windows®.

## RESULTS

Bone resorption was observed 3 weeks after MFS. ABL was significantly greater in the operated area (BL) (Student *t* test;  $p < 0.01$ ) when compared to the control side (BR) (Table 1).

TABLE 1 – Alveolar bone loss area (mm<sup>2</sup>) in the buccal and lingual sides (both left and right) of the mandible, in the control (n = 10) and experimental (n = 10) groups, 21 days after mucoperiosteal flap surgery.

		95%CI	
	Mean	Lower limit	Upper limit
BR area (control)*			
Control	0.41	0.07	0.75
Experimental	0.34	0.16	0.52
Total	0.37	0.10	0.64
BL area (test)*			
Control	0.60	0.14	1.06
Experimental	0.62	-0.16	1.40
Total	0.61	-0.01	1.33
LR area			
Control	0.93	0.41	1.45
Experimental	0.98	0.62	1.34
Total	0.96	0.54	1.38
LL area			
Control	1.05	0.49	1.61
Experimental	0.96	0.40	1.52
Total	1.00	0.46	1.54

95%CI = 95% confidence interval; BR = buccal right; BL = buccal left; LR = lingual right; LL = lingual left.

\* Statistically significant difference between the operated area (BL) and the control area (BR) (Student *t* test).

Bone loss did not vary significantly when EG and CG were compared (Figure 2). Bone loss was observed (CG vs. EG, respectively) in BL:  $0.60 \pm 0.23$  mm<sup>2</sup> vs.  $0.62 \pm 0.39$  mm<sup>2</sup> ( $p = 0.91$ ); in BR:  $0.41 \pm 0.17$  mm<sup>2</sup> vs.  $0.34 \pm 0.09$  mm<sup>2</sup> ( $p = 0.69$ ); in LL:  $1.05 \pm 0.27$  mm<sup>2</sup> vs.  $0.96 \pm 0.28$  mm<sup>2</sup> ( $p = 0.47$ ); and in LR:  $0.93 \pm 0.26$  mm<sup>2</sup> vs.  $0.98 \pm 0.18$  mm<sup>2</sup> ( $p = 0.59$ ) (Figure 3).

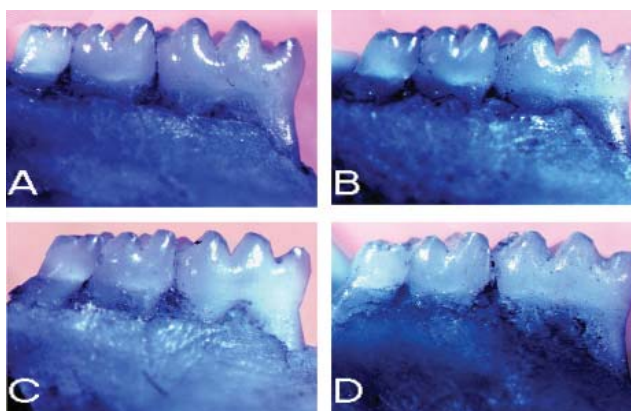


FIGURE 2 – Alveolar bone loss: A) left side, lingual, placebo group; B) left side, lingual, experimental group; C) left side, buccal, placebo group; D) left side, buccal, experimental group.

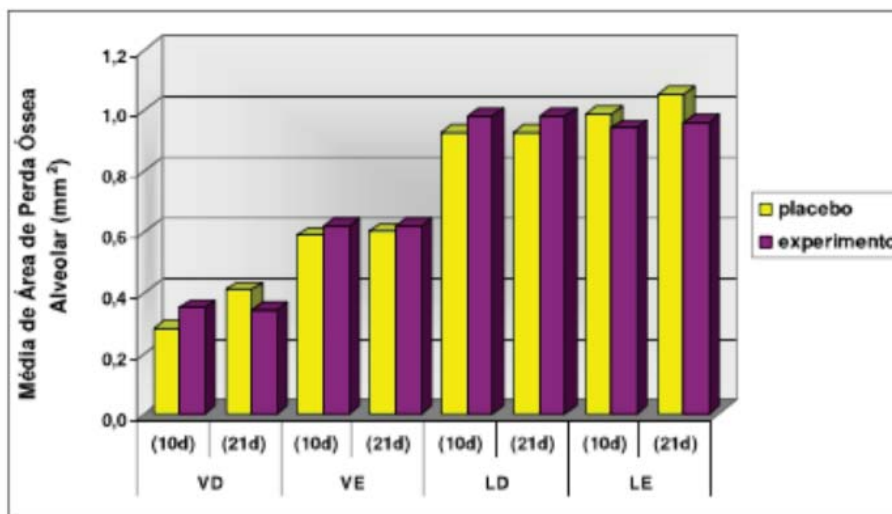


FIGURE 3 – Mean alveolar bone loss (in mm<sup>2</sup>) in the control and experimental groups, right and left aspects, lingual and vestibular sides, 10 and 21 days after mucoperiosteal flap surgery. BR = buccal right; BL = buccal left; LR = lingual right; LL = lingual left.

No weight variation was observed in animals throughout the experimental period.

## DISCUSSION

The present study evaluated the effect of raloxifene in terms of inhibiting ABL after MFS in mice. Under the experimental circumstances here described, raloxifene was not able to prevent or even reduce bone resorption. To our knowledge, this is one of the first studies to assess whether raloxifene affects bone resorption using a rodent model in male mice.

In the present study, an experimental model of MFS was used to induce ABL in mouse mandibles, as different studies have reported bone resorption due to bone exposure by flap displacement (3,5,7). Yaffe et al. (4) conducted a study with rats and observed considerable bone remodeling after MFS in maxillary bones. In mice, this process is sex-independent (5). These studies are in agreement with the results shown in the present experiment, as ABL was significantly greater in the operated side (BL) when compared to the control side (BR).

Bone resorption may interfere negatively with the success of oral rehabilitation therapies. Therefore, the search for drugs and treatments that can prevent or minimize ABL is an important field of periodontal research. Raloxifene, a selective estrogen receptor modulator used in osteoporosis treatment and prophylaxis was tested for its role in bone resorption. The drug mimics the effects of estrogen in the bone, with the

added benefits of not stimulating the uterine endometrium and reducing the risk of breast cancer and the incidence of coronary events and stroke (26-28). *In vitro*, raloxifene plays a role in the modulation of bone homeostasis by inhibiting osteoclastogenesis and bone resorption, stimulating osteoblast activity, and inhibiting interleukin 1 $\beta$ , interleukin 6 and TNF- $\alpha$  (29). *In vivo* studies also demonstrate positive effects of the drug on the maintenance of mineral bone density of the lumbar spine, femoral neck (17,18,28) and tibia (30). Those studies also reported a decrease in biochemical markers of bone remodeling, such as osteocalcin, alkaline phosphatase, interleukin 1 $\beta$ , and interleukin 6 (17,18), as well as a reduced risk of microfractures (28). There is evidence that the efficacy of raloxifene is similar in the bones of male rats and ovariectomized female rats (15,31), with an increased expression of selective bone turnover markers in ovariectomized female rats during the alveolar healing process (20). Conversely, raloxifene replacement has proved unable to recover the estrogen-deficient states (20,32). Thus, the action of the drug does not depend on sex.

Even though the effects of raloxifene in bone metabolism have been widely recognized, the results obtained in the present study have shown that the drug does not prevent MFL-induced ABL. This result may be explained by the evidence suggesting that bone mineral density of spine and hip bones cannot be used as predictors of maxillary bone density (33,34). The hypothesis of site-specific bone remodeling formulated by Podenphant and Engel (35) may also help interpret the results of the present study. Those authors investigated 24 different sites of the skeleton and observed that bone formation undergoes considerable regional variations. This hypothesis was confirmed by Verna et al. (36), who compared the parameters of bone remodeling of the mandible with those of the iliac crest. Titanium implants represent a good example of site specificity in bone remodeling, with data showing that 76% of failure in orthopedic implants occur in patients with osteoporosis (37), while the risk of failure in dental implants does not seem to be higher for patients with osteoporosis (38).

The effects of other drugs, such as bifosfonates and doxycycline, in ABL have been investigated previously. Bifosfonates, drugs prescribed in the treatment and prevention of osteoporosis, have been shown by several studies to decrease ABL after MFS in rats (4,10,12). The results found by Yaffe et al. (4,12) demonstrate a decrease in ABL after bifosfonate therapy. Doxycycline was also shown to be able to prevent MFS-induced ABL in a rat model, according to Grevstad (9). Nevertheless, it is important to carefully consider the methodology adopted in the evaluation of ABL in each study.

Different methods have been proposed to study ABL in animal models, with some methodologies evaluating the mineralized matrix (12-14,39,40), others evaluating histological sections (9,10), and others evaluating linear distance measurements (39-41). Yet, all these methods were shown to have limitations (12,39,40). Yaffe et al. (14) demonstrated the effect of alendronate on preventing ABL using high resolution X-ray microradiography analysis. However, no significant differences were observed when ABL was assessed using measurements of the exposed root area.



Further studies are needed to assess the effect of raloxifene in mandibular bones in terms of bone mineral density, decrease in bone turnover biomarkers, or positive changes in cell counts. Further knowledge of these aspects is relevant for the aim of maintaining alveolar bone.

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